The Separation and Quantitative Determination of the Component Sugars of Mucoproteins

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In recent years increasing attention has been given to a class of carbohydrate-protein complexes, largely because of the biological activity associated with them (gonadotrophin, thyroglobulin, blood-group substances, inhibitors of influenza-virus haemagglutination, Castle's intrinsic factor, etc.). In many of these the carbohydrate moiety represents analytically a minor portion only, though, as in virus inhibitors, this minor portion carries the functional group (Gottschalk, 1954). This subclass of complexes, in which protein predominates, may for convenience be referred to as mucoproteins.

In any quantitative analysis of the carbohydrate moiety of mucoproteins three major difficulties arise: (1) in most cases, if not all, the carbohydrate is a mixed polysaccharide; (2) pure samples of the material to be analysed are usually available in small amounts only; (3) the amino acids liberated on hydrolysis of the mucoprotein are known to interact with sugars. Though colorimetric methods, such as the orcinol (Tillmans & Philippi, 1929), carbazole (Dische, Shettles & Osnos, 1949) and anthrone (Dreywood, 1946) methods, are available for the overall estimate of the non-amino sugars of mucoproteins, there is a demand for a method allowing the quantitative determination of the individual sugars in mucoproteins.

The application to the intact mucoprotein of the orcinol, carbazole and cysteine reagents by Sørensen & Haugaard (1933), Gurin & Hood (1939) and Dische (1947) respectively for the quantitative determination of the component sugars derives from the observation that the absorption curve of the coloured compound formed varies with the individual sugar. With increasing complexity of the mixture, however, an unequivocal interpretation of the curves becomes more and more difficult (Meyer, 1938; Gurin & Hood, 1939).

An alternative method would be to hydrolyse the mucoprotein gently, to separate the liberated sugars from the amino acids and to identify and estimate the individual sugars by chromatography. This technique was first applied to the quantitative analysis of the carbohydrate of a mucoprotein prepared from human urine (Gottschalk, 1952*a, b*). The method has been further developed; the efficiency of each step has been tested and, as

described below, in satisfactory operation in this Laboratory for some time. With this method sugar added to a protein is recovered almost quantitatively; the intrinsic sugar of a mucoprotein is recovered to an extent of 70–90% of that estimated by an independent technique to be present in the original material; the degree of recovery depends upon the type of mucoprotein. As early as 1948, Flood, Hirst & Jones (1948) applied paper partition chromatography to the quantitative analysis of the component sugars of polysaccharides. Since the material analysed by the authors did not contain protein, the main difficulty—the interference of amino acids—did not arise. Glegg, Eidinger & Leblond (1953) and Dixon (1955) have used ion-exchange resins to hydrolyse mucoproteins and simultaneously to separate, by adsorption to the resin, the amino acids from the sugars, which in turn are analysed chromatographically. Even with a soluble mucoprotein (ovomucoid) the recovery in the neutralized hydrolysate was only 61%, and further losses occurred in the later stages, the sugar in the original and after processing being checked by the same method (Dixon, 1955). For insoluble mucoproteins (reticular fibres) no attempt has been made to estimate the recovery (Glegg *et al.* 1953).

EXPERIMENTAL

Materials

Mucoproteins. Bovine serum albumin was a preparation obtained from Armour Laboratories, Chicago. This was used to imitate the protein moiety of a mucoprotein in experiments designed to test the recovery of known amounts of sugars in artificial sugar-protein mixtures. Though bovine serum albumin contains a small amount of intrinsic sugar (0.3%, by orcinol), it was selected for this purpose because it is a well-defined protein. Thyroglobulin was kindly prepared by Professor V. M. Trikojus and Dr M. McQuillan, Melbourne University, according to the procedure of Derrien, Michel & Roche (1948). Ovomucoid was prepared according to Mörner (1894). The homogeneous bovine-submaxillary-gland mucoprotein (BSM) was prepared according to Curtin & Pye (1955). All proteins were stored *in vacuo* and kept for 24 hr. over P_2O_5 before use.

Yeast nucleic acid. A commercial preparation was purified according to the procedure of Smith & Markham (1950).

Sugars. The sugars used were standard preparations, exhibiting with one exception a single spot when tested chromatographically. The sample of chondrosamine contained a small proportion of glucosamine. In experiments with the addition to protein of small amounts of sugar a 0.20% solution of the appropriate sugar was prepared and a portion taken. We are indebted to Professor H. O. Fischer, University of California, Dr H. G. Fletcher, National Institute of Health, Bethesda, Professor W. T. J. Morgan, F.R.S., London, and Dr D. J. Bell, Edinburgh, for generous gifts of rare sugars.

Ion-exchange resin. Dowex 50 (200–400 mesh) was prepared for use in the hexosamine determination according to Boas (1953). When applied for the removal of amino acids before the chromatographic analysis of the sugars, it was necessary to wash the regenerated column until the effluent was completely free of acid.

Analytical methods

Determination of total carbohydrate (non-amino sugars). The overall recovery of the intrinsic sugars in mucoproteins by the method described was checked by using an independent procedure to determine the total carbohydrate in the original material. Of the various methods available the orcinol method appears to be least susceptible to interference by the protein components (Gurin & Hood, 1939; Dische *et al.* 1949). To test this point further the following mixtures were submitted to the orcinol method: 0.15 mg. of D-glucose + 3.6 mg. of bovine serum albumin, 0.15 mg. of soluble starch + 3.6 mg. of bovine serum albumin, 0.05 mg. of D-glucose + 0.05 mg. of cysteine hydrochloride + 0.05 mg. of DL-tryptophan; each mixture present in 1.0 ml. of water. The procedure described by Hewitt (1937) was used, the colour intensity being measured in a photoelectric colorimeter fitted with a green filter (510 m μ). The values obtained coincided within the experimental error ($\pm 3\%$) with the sugar controls (correcting for the intrinsic sugar of bovine serum albumin), indicating non-interference by the nitrogenous components tested. The result of total carbohydrate (non-amino sugar) obtained by the orcinol method is referred to as the 'orcinol value'.

It may be mentioned that with thyroglobulin and a mucoprotein to be discussed in a subsequent paper the total carbohydrate was determined both by the orcinol method and the tryptophan method in the modification by Badin, Jackson & Schubert (1953). Though in the two colour reactions the conditions of sugar degradation and the coloured compounds formed differed, the results agreed within 6%. In all these tests the standard was a sugar mixture whose composition was derived from a preliminary chromatographic run of the processed mucoprotein (see below).

Determination of amino sugars. All samples were hydrolysed with 2N-HCl in a sealed tube at 100° for 6 hr. A longer treatment than is found necessary to obtain a maximum value should be avoided in view of the slight destruction of hexosamine under the impact of hydrochloric acid (Blix, 1948; Strange & Powell, 1954). For quantitative determination of free hexosamine the method of Elson & Morgan (1933) was used. On several occasions the liberated hexosamines, before application of the Elson-Morgan procedure, were purified by adsorption to and elution from a cation-exchange resin (Dowex 50) according to Boas (1953). In all cases the values obtained

by the two procedures closely agreed. N-Acetylhexosamine was estimated according to Morgan & Elson (1934).

Uronic acids. The method of Dische (1947) was used to test for the presence of hexuronic acids.

Paper partition chromatography

General procedure. The technique and solvents used have been described previously (Gottschalk, 1955a). For the detection of hexoses, pentoses and methylpentoses aniline hydrogen phthalate was used (Partridge, 1949). For routine purposes butanol-pyridine-water and butanol-acetic acid-water were preferred as solvents. Amino sugars were located by the Elson-Morgan reagents as applied to chromatography by Partridge (1948). For quantitative evaluation of the individual sugar an appropriate concentration of the sugar mixture was run together with a serial dilution of the standard, each dilution differing by 20% from the previous one. For aldohexoses and methylpentoses concentrations within the range 0.2–0.4% allowed an estimate to be made with an accuracy of $\pm 10\%$. The optimum concentration range for pentoses was found to be slightly lower. It is essential to bring the concentration of the sugar under investigation within this range, since the sensitivity of the method decreases with increasing concentration. A preliminary run was necessary to provide information about the nature and the approximate concentration of the sugars present. A standardized micropipette was used to deliver 3.5 μ l. of the sugar solution to the paper.

When in the course of the procedure it became desirable to check by an independent method the amount of sugar isolated chromatographically as described above, the sugar was eluted from the paper in the following way: an area, 2 in. $\times$ 1 in., located by a guiding spot, was cut out of the paper. After both ends had been slightly pointed the paper was mounted on a glass capillary, one point touching the tip of a burette containing water, and the other draining into a test tube. The sugar was eluted by irrigation with 2.5 ml. of water over a period of 2 hr., conditions found to be adequate for quantitative elution of the amount of sugar applied. A paper blank of equal size was similarly treated. Since even elaborate pretreatment of the chromatographic paper did not prevent the continual release of orcinol-reacting material, a quantitative sugar estimate of the eluate could only be made by heavier loading of the paper. Instead of 3.5 μ l., up to 40 μ l. of the test solution was applied to the paper, thus reducing the value of the blank to about 5% of that of the sugar eluate.

To obtain an estimate of the approximate proportions of glucosamine and galactosamine, if both proved to be present, the purified hexosamines (see above) were chromatographed with collidine (at 4°) or butanol-pyridine-water as solvent. When the concentration of the individual hexosamine in both assay and standards is brought within the range 0.4–0.8%, a satisfactory resolution of the two compounds is obtained. The R_F values of glucosamine and galactosamine in collidine are 0.32 and 0.26 respectively, and in butanol-pyridine-water are 0.21 and 0.17 respectively.

Identification of sugars. The component sugar may be identified by chromatography of the unknown and a standard set of sugars in the four solvents described by Gottschalk (1955a). When the sugar thus characterized is one commonly found in mucoproteins, this procedure may be regarded as satisfactory for provisional identification.

Identification of amino sugars. Following Gardell, Heljenskjöld & Rochnovlund (1950) the hexosamines were oxidized with ninhydrin to the corresponding pentoses, which were identified by chromatographic analysis. It was found desirable to remove the hexosamines from the acid hydrolysate before carrying out the ninhydrin procedure. This was effected by adsorption to and elution from a cation-exchange resin (Boas, 1953).

Analysis of mucoproteins

The essential steps of the procedure to be described are: (1) acid hydrolysis of the mucoprotein; (2) neutralization and concentration of the hydrolysate; (3) removal of unhydrolysed protein moiety and/or polypeptides by ethanol precipitation; (4) removal of amino acids and small peptides by ion-exchange resin; (5) identification and quantitative determination of the individual sugars by paper partition chromatography.

A comparison at different stages of the orcinol value with that of the original material served as a guide for the elaboration of optimum conditions for quantitative sugar recovery before chromatographic separation of the individual sugars. From preliminary experiments it became evident that a major factor interfering with the quantitative recovery from a mucoprotein of the intact monosaccharides is the interaction between the released sugars and amino acids (Maillard, 1912; Gottschalk & Partridge, 1950; Gottschalk, 1952a). To reduce to a minimum the rate of this detrimental reaction, the method was so designed that at high temperatures (e.g. step 1) the volume of the reaction mixture was kept reasonably large and that during concentration procedures (e.g. step 2) low temperatures were maintained.

Step 1. About 25 mg. of the mucoprotein was digested with 5.0 ml. of 0.25 N- H_2SO_4 . A period of 18 hr. heating at 100° in a sealed tube proved to be optimum; more severe conditions did not result in a greater yield of monosaccharides.

Step 2. The cooled hydrolysate was carefully neutralized to pH 7.0 with N-NaOH, and a drop of acetic acid added to the mixture to safeguard the sugar during the following concentration. This concentration was carried out from the frozen state *in vacuo*, the distilling flask being submerged in an ice bath to prevent any rise of temperature, especially toward the end of the drying process. In this way the mixture was concentrated to approximately 2 ml.

Step 3. Ethanol was directly added to the mixture in the distilling flask to give a final concentration of 95% (v/v). The mixture was then transferred quantitatively to a centrifuge cup and allowed to stand in the refrigerator overnight. The precipitate formed was removed and washed on the centrifuge. To the pooled supernatants was added 50 ml. of water containing one drop of acetic acid, and the mixture was concentrated on a steam bath. To facilitate removal of ethanol without decreasing the volume to less than 8 ml., two portions of 10 ml. of water were added during the latter part of the procedure.

Step 4. The solution (8 ml.; pH about 4.0) was passed through a well-washed Dowex 50 column and followed by 12 ml. of water. The effluent was concentrated from the frozen state to about 2 ml. as described in step 2. The concentrate was carefully adjusted to pH 5.0 with 0.5 N- Na_2CO_3 and evaporated to dryness *in vacuo* at 0°. The

absence of any ninhydrin-positive reacting material in the residue indicated complete removal of amino acids.

Step 5. The dried sugars were taken up in an appropriate amount of water, and samples taken for sugar determination by the orcinol method and for chromatographic analysis (see Methods). The accuracy of the determination of the individual sugar may be increased by eluting the individual sugars (see Methods) and submitting the eluates to one of the standard methods based on the reducing power or colour formation of monosaccharides. The increase in accuracy will justify the additional time-consuming step in special cases only.

RESULTS

Test and application of the method

The efficiency of the method, i.e. the degree of recovery of sugar present in the original material as indicated by an independent method, was tested in a twofold way. First, a known amount of sugar(s) was added to a protein and, after processing as above, the result of the chromatographic analysis was compared with the amount of sugar added. As a further check the chromatographically isolated sugar was eluted and the orcinol value of the eluate compared with sugar added. Secondly, mucoproteins were submitted to the procedure described and the chromatographic result was compared with the orcinol value of the original material.

The results of representative analyses are shown in Table 1. As may be seen, the recovery by chromatography of sugars added to a protein is almost quantitative, whether estimated by visual comparison with the standard or by determination of the sugar eluted from the paper. The recovery of intrinsic sugar of a mucoprotein varies between 70 and 90%.

A complete analysis (representative values) of the carbohydrate moiety of the mucoproteins referred to in Table 1, as obtained by the method described, is given in Table 2. Mention may be made that no hexuronic acid was found in the mucoproteins investigated.

Determination of ribose in purine nucleotides

For comparison a sugar linked to nitrogenous material other than amino acids was determined chromatographically. Purified yeast nucleic acid (0.20 mg.) was digested with 0.25 ml. of N- H_2SO_4 for 1 hr. at 100° in a sealed tube. After neutralization with $\text{Ba}(\text{OH})_2$ the precipitate was removed and washed and the pooled supernatants were dried *in vacuo* at 20°. The residue was extracted with hot ethanol, the extract evaporated to a small volume and chromatographed with appropriate ribose standards. The yield of ribose, as free sugar, was 40 μg . Another 0.20 mg. of nucleic acid was similarly digested, and the percentage of purine bases

Table 1. *Recovery by the chromatographic and orcinol methods of sugar from sugar-protein mixtures and mucoproteins*

Material and wt. (mg.)	Sugar content of original material (mg.)	Final recovery of sugar		
		By orcinol (%)	By chromatography (%)	Orcinol value of eluate from chromatogram (%)
Bovine serum albumin, 25.0	0.08*	—	—	—
Galactose, 0.55; mannose, 1.10; bovine serum albumin, 25.0	1.73	92	90	—
Glucose, 1.20; bovine serum albumin, 25.0	1.28	95	—	90
Thyroglobulin, 25.0	1.20*	95	90	—
Ovomucoid, 23.0	1.90*	82	70	—
BSM, 30.0	0.68*	88	68	—

* Orcinol value (expressed as free monosaccharides).

Table 2. *Analysis of the carbohydrate moiety of some mucoproteins*

Values are expressed as free monosaccharides.

Mucoprotein	Sugar components*			Amino sugars			Amino sugar Non-amino sugar†
	Galactose (%)	Mannose (%)	Fucose (%)	Total (%)	Glucosamine (%)	Galactosamine (%)	
Thyroglobulin‡	1.2	2.7	0.4	4.0	4.0	0	0.85
Ovomucoid§	1.4	4.3	0	14.1	14.1	0	1.71
BSM	0.7	0.2	0.7	10.0	0.8	9.2	4.41

* Values obtained by chromatography.

† See also Ujejski & Glegg (1955).

‡ Orcinol value.

§ See also Stacey & Woolley (1940, 1942).

present determined by chromatographic analysis according to Smith & Markham (1950). Purine bases found were adenine 18 μ g., guanine 22 μ g., equivalent to 42 μ g. of purine-bound ribose (expressed as free pentose). The chromatographic recovery was therefore 95%.

DISCUSSION

A major difficulty in the recovery for analysis of the sugar components of a mucoprotein is the interaction between reducing monosaccharides and amino acids or free amino groups of the protein. Whereas ribose may be easily recovered quantitatively from purine nucleotides without any special precautions (see Experimental), processing of a sugar-protein mixture or complex under the same unrefined conditions resulted in losses up to 80% of the added or intrinsic sugar. This difficulty has been overcome by maintaining low temperatures where high concentrations of the reactants are unavoidable and by the removal of any nitrogenous material before drying the sugars. Though these precautions necessitated several steps, the recovery of sugar added to a protein was almost quantitative. This is an unequivocal test for the efficiency of the procedure in recovering free sugar.

With mucoproteins the intrinsic sugar was recovered to an extent of 70–90%, the orcinol value of the original material being taken as 100%. There is no evidence to suggest that the orcinol method, when applied to intact mucoproteins, gives a value which is appreciably too high, provided that the correct standard as predetermined chromatographically is used. Our experiments with artificial mixtures of sugars and protein or amino acids gave no indication of interference by amino acids in the chromogen formation. The main difference between the artificial mixture and the native mucoprotein is the presence of free monosaccharides in the former and of glycosidically bound sugar residues in the latter. Of the mucoproteins investigated thyroglobulin gave an almost quantitative recovery of non-amino sugars, and BSM a substantially lower recovery. It would appear from Table 2 that a striking difference between the two mucoproteins is the amino sugar/non-amino sugar ratio. We have observed, as may be expected from the standard technique for liberating hexosamine (2N-HCl, 6 hr., 100°), that under optimum hydrolysis conditions for non-amino sugars (0.25N-H₂SO₄, 18 hr., 100°) only two-thirds of the hexosamine are released from the mucoprotein. With ovomucoid it was ascertained that at the end of the

mild hydrolysis the liberated amino sugar was completely deacetylated (see also Dixon, 1955), and it is probable that the same treatment would have also released all acetyl groups from the hexosamine still bound. Unless the hexosamine moiety left is a chitin-like structure, an appreciable proportion of the hexosamine residues will be linked glycosidically to non-amino sugars. The non-amino sugar trapped in this way will escape recovery, at step 5, both by the orcinol method and by chromatography. Such a situation would be analogous to the resistance towards acid hydrolysis of the glucosidic linkage in *N*-acetylmethylglucosaminide after deacetylation has taken place; the charged amino group, by repelling the approach of a proton to the glucosidic oxygen, confers an abnormal stability on methylglucosaminide towards acid (Moggridge & Neuberger, 1938). In mucoproteins the extent to which non-amino sugars will be trapped by this mechanism depends on the frequency of the structure hexosaminyl-non-amino sugar within the polysaccharide moiety. The trapping potentialities suggested by a high amino sugar/non-amino sugar ratio may be partly counterbalanced by a structure in which the hexosamine residues, linked mainly with each other, are engaged glycosidically in only small numbers with non-amino sugar. This may explain the similar percentage recovery by chromatography of the intrinsic non-amino sugars with BSM (68%) and ovomucoid (70%), although the ratios differ widely (4.41 and 1.71 respectively). It seems more than coincidence that, with thyroglobulin, with the previously studied urinary mucoprotein (Gottschalk, 1952*b*; Odin, 1952) and with a mucoprotein present in influenza virus (Ada & Gottschalk, 1956), where a recovery by chromatography of 90, 90 and 85% respectively of the non-amino sugars was achieved, the ratio was less than 1.0. It would thus appear that only where good agreement between the orcinol value of the original mucoprotein and the estimate of the component sugars, liberated by mild hydrolysis under optimum conditions, is obtained, will it be possible to establish the true proportion of the individual non-amino sugars.

With BSM there is a marked discrepancy between the values obtained by the orcinol and chromatographic methods when applied to the material carried through step 1 to step 4 (Table 1, columns 3 and 4). It was also observed that during mild hydrolysis of BSM considerable amounts of soluble and insoluble humin material were formed, only part of which was removed in the subsequent steps. The coloured material derives most probably from sialic acid, present to an extent of 17% in BSM (Werner & Odin, 1952) and known to decompose on mild acid treatment, with the pro-

duction of humin matter (Blix, 1936). The soluble humin material and/or any other chromogen formed from sialic acid during hydrolysis (Gottschalk, 1955*b*) may react with orcinol, but being separated from the sugars on paper will not contribute to the chromatographic estimate.

Our results are not in agreement with the finding (Dixon, 1955) that after hydrolysis of ovomucoid with 0.5*N*-H₂SO₄ for 4 hr. at 100° only 30–40% of the material, estimated as sugar by application of the anthrone or orcinol method to the hydrolysate, can be recovered by chromatography. No difficulties were experienced in recovering by chromatography 70% of the intrinsic sugar, as determined by orcinol, when strict control of the pH throughout and maintenance of low temperatures during concentration were observed.

SUMMARY

1. A method allowing the complete analysis of the sugar components of mucoproteins has been described. The main features of the method are the reduction to a minimum of the detrimental interaction between reducing sugars and amino acids before their separation and the chromatographic identification and determination of the individual sugars.

2. The accuracy of the method is $\pm 10\%$. The recovery by the procedure of known amounts of sugar added to a protein is almost quantitative. The intrinsic sugars of a mucoprotein are recovered to an extent of 70–90%. One factor determining the degree of recovery appears to be the amino sugar/non-amino sugar ratio, a low value favouring a high recovery in the cases tested.

3. Within this limitation a complete analysis of the carbohydrate moiety of thyroglobulin, bovine-submaxillary-gland mucoprotein and ovomucoid is presented.

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The Component Sugars of the Influenza-Virus Particle

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The great amount of information obtained over the last 15 years about the biological activities of the influenza virus (Burnet, 1951) stimulated at an early stage detailed biophysical and biochemical investigation of this organism. Thus the particle size and shape, electrochemical behaviour (Stanley, Knight & De Merre, 1945) and enzymic properties (Gottschalk, 1954) have been intensively studied. The virus particle is known to contain four main constituents: protein, lipid, carbohydrate and nucleic acid. Taylor (1944) found for the A strain (PR8) of influenza virus a total carbohydrate content of 12.5% (orcinol method with glucose as standard), made up, on the basis of the carbazole reaction, of mannose or glucose-galactose, or both. According to Knight (1947) the total carbohydrate of the PR8 strain, as determined by orcinol with glucose as standard, is about 7%. Of this, ribose was considered to constitute approximately one-tenth, the remainder being incorporated in a polysaccharide consisting of galactose, mannose and hexosamine, as indicated by the carbazole and Elson-Morgan reactions. The identification by Taylor and Knight of the constituent hexoses is based on a technique involving, for a mixed polysaccharide, the interpretation of superimposed absorption curves. The uncertainty of such an interpretation has been previously discussed (Gottschalk & Ada, 1956). For hexosamine no attempt at identification was made. The pentose nature of the nucleic acid sugar was ascertained by

Bial's orcinol reaction, and its identity as ribose inferred from the observation that a sample of isolated nucleic acid was degraded by ribonuclease. Recently, the pentosenucleic acid content of the virus has been reinvestigated with improved techniques and a value of about 1% for highly purified and infective PR8 virus obtained; no significant amount of deoxyribosenucleic acid was found (Ada & Perry, 1954). In the preceding paper (Gottschalk & Ada, 1956), a method has been described allowing the identification and determination in mucoproteins and nucleic acids of the individual sugars after their separation from the non-carbohydrate moiety and after their chromatographic isolation. The desirability of having accurate information about the nature and amount of the sugar components of the influenza virus prompted a reinvestigation of the carbohydrate of the virus particle with the new technique.

MATERIALS AND METHODS

Influenza virus. The strain A (PR8) was used throughout; it was purified by specific adsorption to and elution from human erythrocytes, followed by two cycles of differential centrifuging in an ultracentrifuge. The purity of the virus preparation was judged by examination in the electron microscope and assessed by determination of the ratio haemagglutinin units: mg. dry wt. Details have been described previously (Ada & Perry, 1954).

Virus nucleic acid. The defatted, dried virus was extracted with hot 10% (w/v) NaCl solution and the nucleic